THE CULTIVATION OF A CELLULOSE-DIGESTING FLAGELLATE, TRICHOMONAS TERMOPSIDIS, AND OF CERTAIN OTHER TERMITE PROTOZOA [1]

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Introduction

The cultivation of some of the intestinal wood-feeding flagellates of termites, known to be necessary for the existence of their host on a cellulose diet (Cleveland, 1924), was attempted with three main purposes in mind. These were: (1) to find what inorganic environment was suitable for these highly specialized protozoa; (2) to obtain in culture for the first time a strictly cellulose-feeding protozoön and to study its nutritional requirements; (3) to discover in what way the action of the protozoa on cellulose furnishes food to their insect host.

For most of the work the large Californian termite, *Termopsis angusticollis,* served as the source of material, although the eastern termite, *Reticulitermes flavipes,* and the roach, *Cryptocercus punctulatus,* were also used. The intestinal fauna of *Termopsis angusticollis* consists of seven species of flagellates of which three, *Trichonympha campanula, T. collaris* and *T. sphærica,* are hypermastigotes, while the other four, *Trichomonas termopsidis, Tricercomitus termopsidis, Hexamastix termopsidis,* and *Streblomastix strix* are polymastigotes. All except the last three feed regularly on cellulose.

Culture Experiments

By means of preliminary experiments conducted in culture cell slides, I found that the most favorable osmotic pressure for the protozoa of *T. angusticollis* is that of a 0.3 to 0.4 per cent sodium chloride solution, that the favorable pH range is 6.8 to 7.2, that a ratio of 97 equivalents of Na

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ion to three of Ca is an optimum one, and that an excess of Na over K is more favorable than the reverse.

Many balanced salt solutions were made conforming more or less to these requirements. The anions used in most of the solutions were chloride, phosphate, bicarbonate, and citrate. Those solutions in which the protozoa were able to survive in good condition for at least a day were then used, with certain nutrients added, for actual culture experiments.

In the setting up of a culture experiment, test tubes provided with a small amount of powdered cellulose and a little Merck's powdered animal charcoal were used. The cellulose was prepared by dissolving Whatman filter paper No. 40 in Cross and Bevans' reagent (1 part by weight $ZnCl_2$, 2 parts concentrated HCl) and reprecipitating in water. The precipitated cellulose was filtered off on a large Buchner funnel, washed with tap water till free of chloride and then with distilled water, and allowed to air-dry. It could then be easily ground up in a mortar to a fine white powder containing many particles small enough to be ingested even by the smallest cellulose-feeding flagellates. The tubes holding the cellulose and charcoal were sterilized either in the autoclave or by dry heat. Each tube then received approximately 8 cc. of the liquid medium, which had been previously sterilized by filtration through a Berkefeld N filter. The liquid in the tubes was covered with sterile vaseline, which effectively prevented any rise in pH due to the escape of CO_2 from the medium.

In most cases, each tube was inoculated with the entire hindgut of a termite. The termite was first disinfected on the outside by a 15 to 40-minute immersion in 1:1000 $HgCl_2$ (this did not injure the protozoa) followed by washing in 95 per cent alcohol and in sterile water. The gut was removed with sterile instruments and placed in the culture medium. The liquid was then again covered with sterile vaseline or left exposed, depending on the particular experiment. The tubes, kept at room temperatures in a large closed cupboard, were examined only once or twice a week, since the growth of the protozoa, if any, was always slow.

By these methods a medium (Solution *A*, Table I) was found in which, with the addition of the proper nutrients, three termite flagellates have been cultured for over three years. Two of these, an as yet undescribed *Trichomonas* from *R. flavipes*, and *Tricercomitus termopsidis*,[3] do not require cellulose. The third, *Trichomonas termopsidis*, must

[3] Full details concerning the cultivation of *Tricercomitus termopsidis* and its method of "encystation" are given in a separate paper to appear in *Arch. f. Protistenkunde*.

have cellulose and small amounts of blood serum. Cultures of this organism were used in several experiments described in the second part of this paper.

Of the hypermastigotes, only *Trichonympha sphærica* was able to survive several days in Solution *A* plus Loeffler's dehydrated blood serum (.001 to .2 per cent) with cellulose and charcoal, but no multiplication took place. A series of changes in the salt composition of Solution *A* led to a solution in which (with .001 per cent Loeffler's blood serum, cellulose, and charcoal) *Trichonympha sphærica* lived several weeks and multiplied slightly. This solution was tried with a variety of nutrients (casein, peptone, amino acids, nucleic acid, etc.) and under a variety of physical conditions (as in collodion bags surrounded by large volumes of sterile culture fluid), but no improvement

TABLE I

Salt	Grams per liter distilled water	
	Solution A	Solution U
$NaCl$	1.169	2.164
$NaHCO_3$	0.840	0.773
$Na_3C_6H_5O_7.2H_2O$ (citrate)	2.943	1.509
$NaH_2PO_4.H_2O$	0.690	0
KCl	0.745	0
KH_2PO_4	0	1.784
$CaCl_2$	0.111	0.083
$MgSO_4$	0	0.048

was effected. Further changes in the salt composition finally gave Solution *U* (Table I) in which (with .01 per cent Loeffler's blood serum, cellulose, and charcoal) good initial cultures of *T. sphærica* were obtained. The organisms were usually most abundant two weeks after inoculation of the tube and grew best when the liquid was not covered with vaseline. At the time of maximum population the pH of the cultures was 7.4. After the second week the pH continued to rise, while the number of protozoa decreased slowly, some surviving as long as six weeks. Subcultures, made with 0.5 to 1 cc. of material, showed fair growth in a small percentage of cases, but no second subculture could be effected. Slight changes in the composition of Solution *U* and in its pH, the use of nutrients other than Loeffler's blood serum, and variations in the degree of exposure to air all failed to produce any improvements. Excellent multiplication in many of the initial cultures and in a few of the first subcultures, but never any multiplication in any of the

second subcultures, suggested the possibility that some essential substance present in the termite's gut is either used up or removed by dilution. Accordingly, a number of termites were defaunated by oxygenation (Cleveland, 1925) and a series of first and second subcultures was then made, with each tube receiving a gut aseptically removed from one of the defaunated termites. Few good first subcultures and no second subcultures were obtained.

It is nevertheless interesting that an organism as highly specialized, both morphologically and physiologically, as *T. sphærica* should have been able to live and multiply to a considerable extent in a relatively simple culture medium. It is also noteworthy that in the case of the three polymastigotes as well as in the case of the hypermastigote, the salt composition of the medium is of the utmost importance. Thus *Trichomonas termopsidis* could be cultured continuously only in Solutions *A* and *U* and not in several other very similar media. Moreover, although Solutions *A* and *U* differ from each other only in the presence of $MgSO_4$ and of more phosphate and less citrate in the latter, yet these small differences were sufficient to make all the difference between mere survival of *Trichonympha sphærica* in *A* and active multiplication in *U*.

Trichomonas termopsidis in Culture; its Nutrition and its Action on Cellulose

The xylophagous flagellate *T. termopsidis* has been maintained in culture since October, 1930, in Solution *A* with 0.2 per cent Loeffler's blood serum, cellulose, and charcoal. The liquid in each tube is kept covered with a layer of vaseline except for a short time when the vaseline seal is broken to permit examination. The pH of the medium, originally 7.0 to 7.2, never goes below 6.8, regardless of the age of the culture.

Subcultures are made from parent cultures two, three, or four weeks old, using about 0.3 cc. of material to a tube. The inoculum is removed from the very bottom of the culture, for the flagellates are rarely present more than a quarter of an inch above the bottom. Before the use of charcoal was begun, growth failed to appear in some of the subcultures, but ever since then the subcultures have been completely successful. In most subcultures growth is slow during the first week, reaching a value of 0.2 to 1 organism per low-power field (10 such fields counted in a sample of one drop from the bottom of the tube). At the end of the second week from 1 to 5 organisms per field are present, and at the end of the third week from 8 to 20. After the third week the cultures will remain in a stationary state for a week or even several weeks, after which time the organisms begin to die off. There were

considerable variations from this usual state of affairs. Some few cultures never got beyond one or two organisms per field, while in others there might be fifty or more.

The *Trichomonas termopsidis* in a culture, up to the time of the fourth or fifth week, are nearly all very active and perfectly normal in appearance, and their bodies contain varying numbers of cellulose particles. Most of the flagellates are from 30 to 50 μ long, although in very young cultures, where division forms are numerous, the smaller organisms predominate. In some old cultures, giant forms and multiple-fission forms have been encountered which can ingest very large pieces of cellulose. All the cultural forms are so obviously like those seen in termites that no detailed morphological study of them has been made. (For the morphology of *T. termopsidis* see Andrews, 1925, and Kirby, 1931.)

Trichomonas termopsidis appears to require very little, if any, oxygen. Not only do the organisms grow best under a vaseline seal, but good growth could also be obtained in tubes connected by means of paraffined stoppers and glass tubing to other tubes containing alkaline pyrogallol, thus creating anærobic conditions. Moreover, exposure of the organisms to atmospheric oxygen is toxic. Thus, when exposed to air in a thin layer of culture fluid in a moist chamber the flagellates rapidly round up and die, while if similarly exposed to nitrogen they continue to swim about actively for several days. A current of air bubbled through a culture at the rate of 60 bubbles per minute killed all the protozoa in less than 24 hours, while a current of nitrogen bubbled through another portion of the same culture at the same rate had no effect.

A bacteriological study of the cultures of *T. termopsidis*, made when the strains were several months old, revealed that only one species of bacteria (derived from the termite's gut) was present. This organism, a short Gram-negative bacillus, grows well at room temperature in broth, as well as in Solution *A* with only $(NH_4)_2SO_4$ as its source of nitrogen. It ferments glucose with gas production, but it does not attack cellulose or cellobiose and cannot live in media ordinarily used for cellulose-fermenting bacteria. Many attempts have been made, using a variety of methods, to free the cultures of this last contaminating organism, but none was successful.

Although the ideal of a bacteria-free culture has thus not been attained, it has nevertheless been possible to perform some experiments concerning the nutrition of *Trichomonas termopsidis*. In one experiment, subcultures were made from healthy and comparable parent cultures growing in the standard medium into media in which the Loeffler's

dehydrated blood serum was replaced by 0.1 per cent Difco desiccated blood serum, 0.1 per cent beef broth, 0.1 per cent Bactopeptone, 0.1 per cent cystine plus 0.2 per cent glycine, 0.01 per cent cystine plus 0.01 per cent glycine plus 0.01 per cent tyrosine, 0.1 per cent asparagin or 0.1 per cent Difco hydrolyzed blood serum. Growth occurred only in media containing blood serum or hydrolyzed blood serum. In other series of experiments the cellulose was replaced by rice starch, dextrin, inulin, glycogen, cellobiose, agar, cellulose tri-acetate, or cellobiose octa-acetate. No growth of the flagellates took place, although controls containing cellulose always showed excellent growth. *Trichomonas termopsidis* in

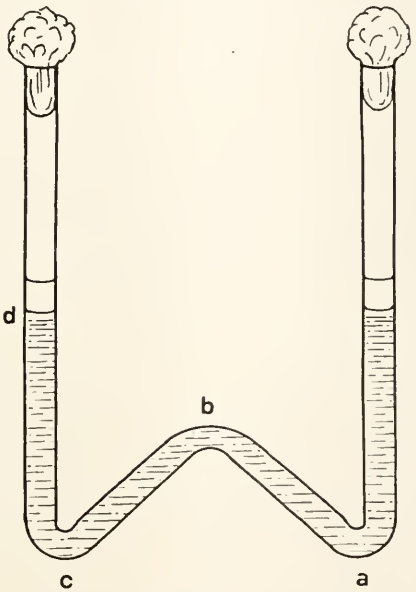

FIG. 1. Diagram of M-tube.

culture appears to be specific for cellulose as its carbon source. In this respect it, like most of the other symbiotic flagellates of termites, closely resembles certain bacteria, such as *Spirochæta cytophaga* (Hutchinson and Clayton, 1919) and *Bacillus cellulosæ-dissolvans* (Khouvine, 1923), which are likewise limited to cellulose as their carbon source.

The maintenance of *Trichomonas termopsidis* for several years on a cellulose diet, its inability to live in culture when the cellulose is replaced by other polysaccharides, and the extraction from the cultivated organisms of a cellulase (Trager, 1932) prove that this flagellate is capable of digesting cellulose.

Since the bacillus present in the cultures of *Trichomonas termopsidis* is a very active glucose fermenter, no hope was entertained of being able to demonstrate unequivocally the production of glucose by cultures of the flagellate. Tests with Benedict's solution of the fluid of active cultures were always negative. Some very strong indirect evidence for glucose production was, however, obtained. If an active culture of the trichomonads was divided into two equal parts, of which one was heated to 43° and held there for 10 minutes, thus killing all the protozoa without injuring the bacteria, and if each portion was then placed in a fermentation tube or in a test tube with a heavy layer of vaseline over it, then, after the lapse of several days, gas appeared only in the tube with the live protozoa. To determine whether this gas was produced by the bacteria from glucose formed through the activities of the protozoa, or whether it was produced directly by the protozoa, the following experiment was performed. Twenty active cultures of *T. termopsidis* were mixed in a large sterile tube and centrifuged. The supernatant liquid, determined by microscopic examination to be free of protozoa, was removed completely, and then the residue, containing the protozoa, was resuspended in 5 cc. of this supernatant liquid. Four tubes, shaped like inverted M's (Fig. 1), made from 6 mm. glass tubing and previously plugged with cotton and sterilized, were now set up as follows. Numbers 1 and 2 received each two pipettefuls, and Nos. 3 and 4 three pipettefuls, of the protozoa-free supernatant liquid. The tubes were manipulated so that no air-bubbles remained in them. To one arm, suitably marked, of Nos. 1 and 3 was then added one pipetteful of the protozoan suspension, very carefully and in such a manner that the protozoa settled in notch *a* of the tube and did not rise to the level of notch *b* (Fig. 1). The remainder of the protozoan suspension was then heated to 43° C. and kept at this temperature for 10 minutes. One pipetteful of this dead suspension was then added to one arm of Nos. 2 and 4. The liquid in both arms of all four tubes was covered with a heavy layer of vaseline. In such an arrangement, it is evident that most of the gas formed should accumulate at notch *b* and that, if the protozoa cannot swim up over notch *b*, then any gas appearing in notch *c* or under the vaseline at *d* (Fig. 1) could not possibly have been formed by the protozoa. Within one week after the date of setting up the experiment, in both tubes 1 and 3 gas was present at notch *c* as well as at notches *a* and *b*. Microscopic examination of samples removed from notches *a* and *c* showed that protozoa were present only in notch *a*. In tubes 2 and 4 there was no gas whatever. This proves conclusively that, in

tubes 1 and 3, the protozoa present in notch *a* produced by their action on cellulose a soluble substance which diffused over toward notch *c* and was there fermented by the bacteria. That this substance was glucose seems almost certain, in view of the facts that glucose and cellobiose are the only simple sugars formed from cellulose and that the bacillus present readily ferments glucose with gas production, but cannot utilize cellobiose. That this glucose was not produced as the result of the extra-cellular action of cellulase is indicated by the fact that, although cellulase could be demonstrated in the supernatant fluid of old degenerating cultures, it could not be demonstrated in the supernatant fluid of active healthy cultures such as were used for the experiment detailed above. It thus appears that *T. termopsidis* and other symbiotic intestinal flagellates ingest cellulose, digest it to glucose, and then excrete part of the glucose, sharing it with their insect host.

Summary

By means of preliminary experiments, the osmotic pressure, pH, and mono- to bivalent ion ratio most favorable to the survival of the symbiotic intestinal flagellates of termites were determined. On the basis of these and other facts, balanced salt solutions were constructed and tested, with the addition of cellulose and low concentrations of protein, as culture media.

In this way a medium was obtained in which the xylophagous flagellate, *Trichomonas termopsidis,* from *Termopsis angusticollis,* has been cultured for over three years. A *Trichomonas* from *Reticulitermes flavipes,* and *Tricercomitus termopsidis* from *Termopsis angusticollis* were cultured in the same medium. The last two organisms did not require cellulose.

In a somewhat different medium, excellent initial cultures of the hypermastigote, *Trichonympha sphærica,* from *Termopsis angusticollis,* were obtained, and the organisms could be carried through a first subculture but not through a second. Attempts to improve the medium so as to secure continuous cultivation failed.

Experiments with *Trichomonas termopsidis* showed that it cannot utilize any carbon source other than cellulose. This protozoön probably secretes glucose, which is used by its insect host.

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